SOURCES AND MECHANISM OF CATALASE ACTIVITY IN THE CATALASE TEST FOR ABNORMAL MILK[1]

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ABSTRACT

Catalase scores of fat and cell-free milk were 25 ± 9% of the total catalase scores of whole milk. Relatively more free catalase was present in mastitic than in normal milk. Addition of H_2O_2 caused death and disintegration of leucocytes and addition of surfactants increased the rate of death of leucocytes and rate of O_2 release from H_2O_2. Disruption of milk leucocytes by insonation released catalase without appreciable loss of activity. From these results it was concluded that, in the catalase test the H_2O_2 caused death and disintegration of leucocytes, thus liberating catalase which released O_2 from H_2O_2. Enzyme activity of beef liver catalase was greater in the presence of heat-labile whey proteins of milk than in buffer. Presence of more than 2 x 10^8/ml of high catalase producing bacteria in milk contributed significantly to the catalase score of the sample.

Although the amount of catalase is known to be increased in milk from cows with mastitis, some poor correlations between leucocyte counts and catalase scores in milk have been reported *(1, 3, 5, 10)*. Spencer and Simon *(12)* found appreciable amounts of catalase in the cell-free fractions of normal and mastitic milk, but little is known about the quantitative distribution of catalase in cellular and cell-free fractions of normal or mastitic milk. The purpose of this report is to present results of some studies which were made to determine the sources of catalase activity in milk, including the effect of the presence of bacteria in milk on the catalase scores and the effect of certain milk constituents on the activity of beef liver catalase.

The mechanism of the catalase test on milk is inadequately understood. To obtain information on this aspect of the test, the effect of insonation, H_2O_2 and surfactants on viability of the leucocytes was studied in relation to the catalase activity of the samples.

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MATERIALS AND METHODS

Milks used throughout these investigations were well mixed bucket-milk samples from individual cows of the University of Wisconsin Dairy herds. All samples were tested within 4 hr after milking. The catalase test was conducted using tube method A *(9)* with 10 ml of milk, 2 ml of 3% H_2O_2, and 3 ml of water, incubated at room temperature for 3 hr with 4 replicates for each sample. Counts of total leucocytes, live and dead leucocytes were made as described elsewhere *(9)*.

Sources of catalase in milk. Portions of samples of milk from 28 cows were centrifuged at 600 x *g* for 10 min, and layers of fat removed and the skimmilk decanted. This was repeated twice for each sample to remove all of the leucocytes from the milk as determined by microscopic examination. Catalase tests were made with uncentrifuged and centrifuged preparations and the data recorded as total and free catalase, respectively.

On the basis of these tests six apparently normal cows whose whole milk had total catalase scores under 30% O_2 and six with histories of chronic mastitis and total catalase scores above 30% O_2 were selected for additional investigations. Bucket-milk samples were obtained at weekly intervals for 8 weeks and total and free catalase scores in each sample were determined.

Effect of bacteria on catalase scores of milk. Cultures of *Streptococcus agalactiae, Staphylococcus aureus, Escherichia coli,* and *Bacillus subtilis* were prepared in brain heart infusion broth (Difco) with overnight incubation at 37 C. A mixed culture of bacteria from milk, containing mainly *Pseudomonas* sp. was similarly prepared. Graded quantities of each culture were added to milk of low catalase scores and bacterial plate count *(11)* and catalase tests were performed on each sample. The significant difference in catalase scores of the same sample with and without the addition of bacteria was determined by the method of least significant difference *(13)*.

Effect of milk constituents on catalase activity. To determine the effect of milk constituents on catalase activity, six samples of milk with catalase scores varying from 4 to 36% O_2 production were divided into 8 portions each. Equal amounts of beef liver catalase were added to each of 4 replicate samples and catalase activity determined. Controls consisted of 4 replicates with equal amounts of beef liver catalase in 0.1 M phosphate buffer of pH 6.6 and 4 replicates each of the milk samples without added catalase.

In further experiments, beef liver catalase was added to phosphate buffer, skimmilk, skimmilk heated at 90 C for 10 min, and "lactalbumin" or "lactoglobulin" prepared as described by Jenness and Patton *(6)*, and catalase tests were performed on each mixture.

Effect of disruption of leucocytes on catalase activity. Portions of four milk samples with catalase scores ranging from 20 to 100% O_2 production, and suspensions of milk leucocytes in phosphate buffer were insonated[1] for 5, 10, and 15 min. Total, live and dead leucocytes, and catalase scores were de-

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TABLE 1. EFFECT OF MILK CONSTITUENTS ON THE ACTIVITY OF BEEF LIVER CATALASE

	Buffer pH 6.6	Skimmilk	Skimmilk heated 90 C for 10 min	Whey	"Lactalbumin" 0.45%	"Lactoglobulin" 0.15%
Per cent O_2 production without added catalase	0	19.5	0.5	2	0.5	1.5
Per cent O_2 production with added catalase	20	47.5	22	31	25.5	31.5
Activity of added catalase in relation to that in buffer[1]	100	140	107	145	125	150

[1]20% O_2 produced by added catalase in buffer was assumed as 100% activity of the beef liver catalase and the others were calculated as follows:

$$\frac{\left[\begin{array}{c}O_2 \text{ production AFTER addition of the catalase} - O_2 \text{ production BEFORE addition of the catalase}\end{array}\right]}{O_2 \text{ produced by the catalase in the buffer}} \times 100$$

termined before and after insonation. The samples which were insonated for 5 min were centrifuged at 800 x g for 10 min, fat and sediment were removed, and catalase scores determined. Total catalase scores of control samples, not subjected to insonation, were also determined.

Effect of H_2O_2 and surfactants on leucocytes and catalase activity. Six milk samples, each of which contained more than 10^6 leucocytes per ml, were centrifuged at 400 x g for 10 min, and the sediment was resuspended in a small volume of the original milk. The above suspension was divided into three 10 ml portions. To one portion, 2 ml of 3% H_2O_2 and 3 ml of water were added and proportions of live and dead leucocytes were determined at 0, 10, 20, 30, 40, 60, and 90 min. To the other two portions of leucocyte suspensions in milk, 2 ml of 3% H_2O_2 and 3 ml of 1:5 Mastest solution[2] or 3 ml of 0.5% sodium lauryl sulfate were added and the proportion of live and dead leucocytes was determined at 0, 10, 20, 30, 40, 50, and 60 min.

In a different experiment, milk samples from 10 cows with catalase scores ranging from 10 to 70% (mean 26%) O_2 production were divided into 12 portions of 10 ml each. The following materials were added to each set of 4 replicate milk samples: 3 ml of 1:5 Mastest solution; 3 ml 0.5% sodium lauryl sulfate; 3 ml H_2O. The catalase test was then performed on each sample by addition of 2 ml 3% H_2O_2. Oxygen released was recorded at 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 min.

activity varied from 15 to 50% (mean 25 ± 9*) of the total catalase score. The correlation coefficient between free catalase scores and total catalase scores was .89 ($P < .05$). The proportion of dead leucocytes in the 28 samples ranged from 5 to 50% (mean 21 ± 12*), of the total leucocytes. The correlation coefficient between free catalase scores and log of the number of dead leucocytes was .59 ($P < .20$).

RESULTS

Sources of catalase in milk. Catalase tests conducted on each sample before and after centrifugation showed that in different samples the free catalase

The total catalase scores of milk from normal cows was less than 30% O_2 production of which 15 to 35% (mean 26) resulted from free catalase. Milk from cows with mastitis had relatively higher free catalase activity, ranging from 25 to 50% (mean 41) of the total catalase scores.

Effect of bacteria on catalase score of milk. The least significant difference in catalase scores was found to be approximately 4% O_2 production. On this basis, significant increases in catalase score occurred when more than 2 x 10^5 *B. subtilis*/ml, 1 x 10^6 *E. coli*/ml, 5 x 10^6 *S. aureus*/ml and 5 x 10^6 mixed culture/ml were present. A significant increase in catalase score was not observed in samples with as many as 5 x 10^6 *S. agalactiae*/ml.

Effect of milk constituents on catalase activity. The increase in O_2 production contributed by the added beef liver catalase in whole milk samples was 46% (range 20 to 60%) greater than the same amount of beef liver catalase in buffer.

As shown in Table 1, the activity of beef liver catalase was 40% greater in skimmilk than in buffer. This increased activity was eliminated by heating skimmilk before adding beef liver catalase. Whey buffered at pH 6.6 (after acid precipitation of casein) had a catalase enhancing effect of the same order as unheated skimmilk. This enhancing activity was associated to a greater extent with "lactoglobulin" than

[1]"Sonifier," Branson Instruments, Stanford, Conn.
[2]Norden Laboratories, Lincoln, Nebraska.
*Standard deviation.

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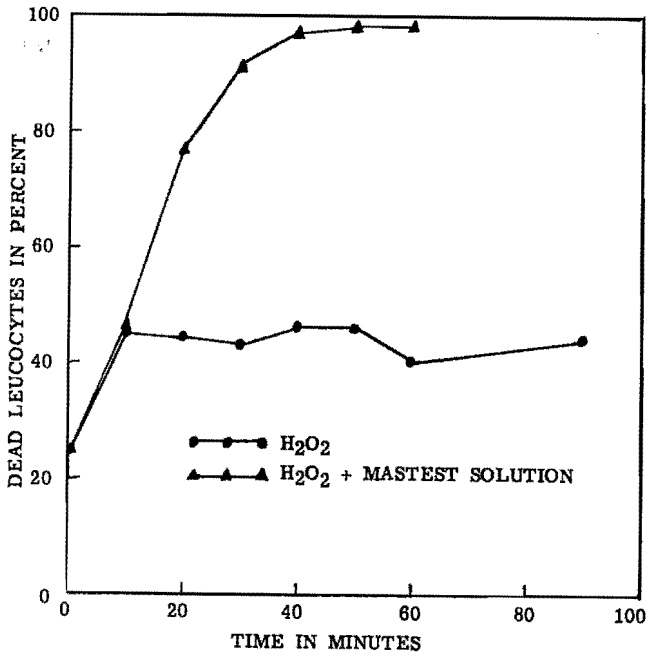

Figure 1. Effect of H_2O_2 and surfactant (Mastest solution) on leucocytes of milk.

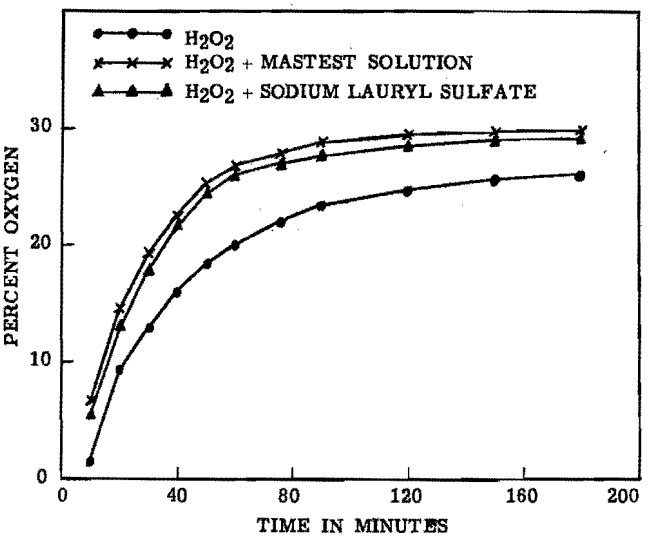

Figure 2. Effect of surfactants on the rate of release of oxygen (average of 10 different samples).

with "lactalbumin." Removal of fat, addition of Fe^{3+}, Ca^{2+}, or Mn^{+} had no significant effect on catalase activity in this system.

Effect of sonic disruption of leucocytes on catalase activity. Leucocyte counts in the samples before insonation ranged from 7.0×10^5 to 1.3×10^7 (mean 4.6×10^6) cells/ml of which 15 to 38% (mean 26%) were dead cells. After insonation for 5 min more than 90% of the leucocytes were disintegrated and non-stainable with methylene blue and of the stainable cells more than 90% were dead, so that less than 1% of the initial leucocytes remained alive. The catalase scores of the samples insonated for 5 min were 90 to 100% of the catalase scores of the untreated samples, and were only slightly less when insonation was extended to 15 min. The catalase scores of the insonated, centrifuged cell-free skimmilk were 90 to 95% of those of the untreated samples.

Effect of H_2O_2 and surfactants on leucocytes and catalase activity. The results are presented in Fig. 1. Proportion of dead leucocytes in the presence of H_2O_2 reached maximum of 45% in 10 min, and the remaining leucocytes appeared to be viable through the 90 min observation period. After 40 min exposure to the surfactants in the presence of H_2O_2 practically all of the leucocytes were dead. Significant differences in rates of death of leucocytes between Mastest solution treated and sodium lauryl sulfate treated samples were not observed.

In the experiment in which catalase scores were determined with and without Mastest solution or sodium lauryl sulfate, the rate of release of O_2 was faster, reaching completion in 90 min in the presence of surfactants. These results are presented in Fig. 2. A statistical comparison of catalase test scores at 90 min in samples with surfactant and 180 min in control samples was made. The correlation coefficient is .99 ($P < .01$) and the regression coefficient is 1.035 (results with surfactants being y). The standard deviations of 4 replicates were 1.9% and 2.8% O_2 production with and without surfactants, respectively.

DISCUSSION

These results confirm the findings of Spencer and Simon *(12)* that catalase may occur free in milk. Relatively greater amounts of free catalase were present in mastitic than in normal milk. The origin of this free catalase is not c l e a r. Although the amount of free catalase was poorly correlated with the number of dead cells in the untreated milk samples, cells which had died and disintegrated in the udder before the milk samples were drawn could not be estimated. It is proposed that variations in the free catalase content of the milk of different cows may be related to variations in the rate of death and disintegration of leucocytes and perhaps parenchymal cells in the udder as part of the inflammatory process. The presence of free catalase in milk could be one of the factors responsible for the poor correlation between cell counts and catalase scores which have been reported *(1, 3, 5, 10)*.

The present experiments on the effect of bacteria on milk catalase scores are in general agreement with those of Monlux *(8)*. Even the most active catalase producing pure cultures of *B. subtilis*, significantly increased catalase scores only when populations were 2×10^8/ml or higher. Since the legal standard in the

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United States for grade A raw milk is not more than 2×10^5 bacteria/ml, and for manufacturing grade raw milk is not more than 1×10^6 bacteria/ml, the contribution of bacteria to the catalase scores of milk is not likely to be significant unless the organisms consist entirely of high catalase producing bacteria.

Hydrogen peroxide inactivates catalase *(6)*, as it is a strong oxidizing agent which denatures proteins. The greater activity of beef liver catalase in milk, skimmilk, and whey than in buffer appear to result from stabilization of the enzyme by heat-labile whey proteins in the presence of H_2O_2. It is reasonable to assume that the natural catalase in milk is similarly stabilized. The whey protein concentration, which varies among different cows *(6)*, in the same cow at different stages of lactation *(2)*, and in mastitic cows depending on the degree of inflammation *(4, 7)*, may influence the catalase scores of milk. However, more experimentation is needed to establish the significance of whey protein concentration on catalase scores of milk.

In the presence of H_2O_2 alone the catalase reaction did not reach completion by 90 min, probably because something less than one-half of the leucocytes did not die and release their catalase. The rate of death of leucocytes in the presence of H_2O_2 alone, as shown in Fig. 1, is subject to considerable variation because of discrepancy of the methods used for counting total leucocytes and live and dead leucocytes. In the methylene blue stained preparations for total leucocyte counts, large fragments of disintegrated leucocytes with stainable nuclei are generally counted as cells, whereas, in the trypan blue staining method for counting the proportion of live and dead leucocytes only the intact whole cells are enumerated. Addition of surfactants increased the initial rate of O_2 release and caused the catalase reaction to reach completion in about 90 min (Fig. 2), because almost all of the leucocytes had died and released their catalase by about 40 min (Fig. 1). Catalase was completely released from leucocytes by insonation without loss of activity.

Results of this study indicate that catalase is an intracellular enzyme and the role of H_2O_2 in the catalase test is to cause death of leucocytes, thus releasing the catalase and then providing substrate for the enzyme.

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